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Citrus Exocortis Viroid RT-PCR Detection Kit

Product Insert

Product# 47600

Pathogen Information

Citrus Exocortis Viroid (CEVd) is a member of the genus *Pospiviroid* within the family *Pospiviroidae*. CEVd is the causal agent of the exocortis disease characterized by a bark shelling or scaling disorder of trifoliate orange used as rootstock. The citrange hybrids and Rangpur lime, both used as rootstocks, are also sensitive and develop bark scaling symptoms and stunting. Viroids are small plant pathogens consisting of a naked single-stranded circular RNA molecule of 246–475 nt, that do not encode any proteins but are endowed with autonomous replication in their host plants. Norgen's Citrus Exocortis Viroid RT-PCR Detection Kit is a rapid and sensitive CEVd detection tool which can be applicable for the early diagnosis of CEVd.

Principle of the Test

Norgen's Citrus Exocortis Viroid (CEVd) RT-PCR Detection Kit constituents a ready-to-use system for the isolation and detection of CEVd using end-point one step RT-PCR. The kit first allows for the isolation of viroid RNA from plant tissues using spin-column chromatography based on Norgen's proprietary resin. The RNA is isolated free from inhibitors, and can then be used as the template in a one step RT-PCR reaction for CEVd detection using the provided CEVd Master Mix. The CEVd Mastermix contains reagents and enzymes for the specific amplification of a 270 bp region of the viroid genome. In addition, Norgen's CEVd RT-PCR Detection Kit contains a second Mastermix, the Control 2X RT-PCR Master Mix, which can be used to identify possible PCR inhibition and/or inadequate isolation via a separate RT-PCR reaction with the use of the provided *PCR control (PCRC)* or *Isolation Control (IsoC),* respectively. This kit is designed to allow for the testing of 24 samples.

Kit Components:

Component	Contents
Lysis Solution	25 mL
Wash Solution	11 mL
Elution Buffer	3 mL
Mini Spin Columns	24
Collection Tubes	24
Elution tubes (1.7 mL)	24
CEVd 2x RT-PCR Master Mix	0.35 mL
Control 2x RT-PCR Mastermix	0.35 mL
Isolation Control (IsoC)*[a]	0.3 mL
CEVd Positive Control (PosC)*[b]	0.1 mL
Nuclease Free-Water	1.25 mL
Norgen's DNA Marker	0.1 mL
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** IsoC = Isolation Control ; PosC= Positive Control*
[a] The isolation control is a RNA transcript product.
[b] The positive control is CEVd RNA transcript

Customer-Supplied Reagents and Equipment

- Benchtop microcentrifuge
- 1.5 mL microcentrifuge tubes
- 96 – 100% ethanol
- 70% ethanol
- Mortar and pestle or other homogenization device

Storage Conditions and Product Stability

- The Positive Control (**CEVd *PosC,*** red cap) and Isolation Control (***IsoC*,** orange cap) should be stored at -70°C. If needed, make aliquots of the controls according to the volume used in the protocol (10 µL of **CEVd *PosC*** or 10 µL of ***IsoC***) prior to freezing.
- The **CEVd 2X RT-PCR Mastermix** (green cap) and the **Control 2X RT-PCR Mastermix** (yellow cap) should be stored at -20°C upon receipt (-70°C for long-term). Make appropriate aliquots and store at -20°C if needed.
- All other kit components may be stored at room temperature
- The **CEVd 2X RT-PCR Mastermix** and the **Control 2X RT-PCR Mastermix**, Positive Control and Isolation Control should not undergo repeated freeze-thaw (a maximum freeze-thaw of three times).
- For RT-PCR
    - Allow reagents to thaw at room temperature prior to use
    - When thawed, mix the components and centrifuge briefly
    - Work quickly on ice
    - After addition of RT-PCR Mastermix use within one hour

General Precautions

The user should exercise the following precautions when using the kit:

- Use sterile pipette tips with filters.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Work quickly on ice.

Quality Control

In accordance with Norgen's ISO 9001 and ISO 13485-certified Quality Management System, each lot of Norgen's CEVd RT-PCR Detection Kit, including the CEVd 2x RT-PCR Master Mix, Control 2X RT-PCR Mastermix, Isolation Control and CEVd Positive Control are tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

Norgen's CEVd RT-PCR Detection Kit is designed for research purposes only.

Product Warranty and Satisfaction Guarantee

NORGEN BIOTEK CORPORATION guarantees the performance of all products in the manner described in our product manual. The customer must determine the suitability of the product for its particular use.

Disclaimers

The **Lysis Solution** contains guanidinium salts, and should be handled with care. Guanidinium salts form highly reactive compounds when combined with bleach, thus care must be taken to properly dispose of any of these solutions.

Safety Information
Ensure that a suitable lab coat, disposable gloves and protective goggles are worn when working with chemicals. For more information, please consult the appropriate Material Safety Data Sheets (MSDSs). These are available as convenient PDF files online at *www.norgenbiotek.com*.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Working with RNA
RNases are very stable and robust enzymes that degrade RNA. Autoclaving solutions and glassware is not always sufficient to actively remove these enzymes. The first step when preparing to work with RNA is to create an RNase-free environment. The following precautions are recommended as your best defense against these enzymes.

- The RNA area should be located away from microbiological work stations.
- Clean, disposable gloves should be worn at all times when handling reagents, samples, pipettes, disposable tubes, etc. It is recommended that gloves are changed frequently to avoid contamination.
- There should be designated solutions, tips, tubes, lab coats, pipettes, etc. for RNA only.
- All RNA solutions should be prepared using at least 0.05% DEPC-treated autoclaved water or molecular biology grade nuclease-free water.
- Clean all surfaces with commercially available RNase decontamination solutions.
- When working with purified RNA samples, ensure that they remain on ice during downstream applications.

Protocol

A. CEVd Total RNA Isolation

Important Notes Prior to Beginning Protocol:

- All centrifugation steps are carried out in a benchtop microcentrifuge at **14,000 x g (~ 14,000 RPM)** except where noted. All centrifugation steps are performed at room temperature.
- A variable speed centrifuge should be used for maximum kit performance. If a variable speed centrifuge is not available a fixed speed centrifuge can be used, however reduced yields may be observed.
- Ensure that all solutions are at room temperature prior to use.
- Prepare a working concentration of the **Wash Solution** by adding 25 mL of 95 - 100% ethanol (provided by the user) to the supplied bottle containing the concentrated **Wash Solution**. This will give a final volume of 36 mL. The label on the bottle has a box that may be checked to indicate that the ethanol has been added.
- Both fresh or frozen samples may be used for this procedure. Samples should be flash-frozen in liquid nitrogen and transferred immediately to a -70°C freezer for long-term storage. Do not allow frozen samples to thaw prior to grinding with the mortar and pestle in order to ensure that the integrity of the RNA is not compromised.
- While the provided procedure does not rely on the use of liquid nitrogen to homogenize the sample, both fresh and frozen tissues can optionally be processed using other homogenization methods, including grinding with liquid nitrogen.
- **Isolation Control (*IsoC*)**
    - An Isolation Control (*IsoC*) is supplied. This allows the user to control the RNA isolation procedure. For this assay, add the Isolation Control *(IsoC)* to the lysate during the isolation procedure
    - The Isolation Control *(IsoC)* must not be added to the sample material directly.

- Do not freeze and thaw the Isolation Control *(IsoC)* more than 2 times.
- The CEVd Isolation Control *(IsoC)* must be kept on ice at all times during the isolation procedure.

- The RT-PCR components of the CEVd RT-PCR Detection Kit should remain at -20°C until RNA is extracted and ready for RT-PCR amplification.
- It is important to work quickly during this procedure.

1. Lysate Preparation

a. Transfer ≤100 mg of plant tissue into a mortar that contains 800 µL of **Lysis Solution**. (The volume of plant tissue and Lysis Solution can be increased proportionally. For instance, 0.5 g of plant tissue requires 4 mL of **Lysis Solution**. Extra lysis solution can be purchased separately. See Related Products table). Grind the sample using a pestle until the tissue is completely macerated.

Note: Other homogenization devices such as Bioreba extraction bag and a homogenizer can also be applied to this procedure.

b. Using a pipette, transfer the lysate into an RNAase-free microcentrifuge tube (not provided).

c. Spin the lysate for 2 minutes to pellet any cell debris. Transfer the supernatant to another RNase-free microcentrifuge tube. Note the volume of the supernatant/lysate.

Note: Ensure that only the clear supernatant is transferred, avoiding any of the debris. If necessary, repeat Step **1c** if visible precipitates are still present after the first spin.

d. Add an equal volume of 70% ethanol (provided by the user) to the lysate collected above (100 µL of ethanol is added to every 100 µL of lysate). Vortex to mix. **Proceed to Step 2.**

2. Binding RNA to Column

a. Assemble a column with one of the provided collection tubes.

b. Add 10 µL of **Isolation Control (*IsoC*)** to the lysate mixture.

c. Apply up to 600 µL of the clarified lysate with ethanol onto the column and centrifuge for 1 minute at **14000 × g (~14,000 RPM**). Discard the flowthrough and reassemble the spin column with the collection tube.

Note: Ensure the entire lysate volume has passed through into the collection tube by inspecting the column. If the entire lysate volume has not passed, spin for an additional minute.

d. Depending on your lysate volume, repeat step **2c** if necessary.

3. Column Wash

a. Apply 400 µL of **Wash Solution** to the column and centrifuge for 1 minute.

Note: Ensure the entire wash solution has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute.

b. Discard the flowthrough and reassemble the spin column with its collection tube.

c. Repeat steps **3a** and **3b** to wash column a second time.

d. Wash column a third time by adding another 400 µL of **Wash Solution** and centrifuging for 1 minute.
e. Discard the flowthrough and reassemble the spin column with its collection tube.
f. Spin the column for 2 minutes in order to thoroughly dry the resin. Discard the collection tube.

4. RNA Elution

a. Place the column into a fresh 1.7 mL Elution tube provided with the kit.
b. Add 50 µL of **Elution Buffer** to the column.
c. Centrifuge for 2 minutes at **200 x *g* (~2,000 RPM)**, followed by a 1 minute spin at **14,000 x *g* (~14,000 RPM)**. Note the volume eluted from the column. If the entire volume has not been eluted, spin the column at **14,000 x g (~14,000 RPM)** for 1 additional minute.
d. The purified RNA sample could be used immediately for RT-PCR as described below. It is recommended that samples be placed at -70°C for long term storage.

B. CEVd RT-PCR Assay Preparation

Notes:

- Before use, suitable amounts of all RT-PCR components should be completely thawed at room temperature, gently vortexed and centrifuged briefly.
- The amount of CEVd 2X RT-PCR Master Mix provided is enough for up to 32 RT-PCR reactions (24 sample RT-PCR, 4 positive control RT-PCR and 4 no template control RT-PCR).
- For each sample, one RT-PCR reaction using the **CEVd 2X RT-PCR Mastermix** and one RT-PCR reaction using **Control 2X RT-PCR Mastermix** should be set up in order to have a proper interpretation of the results.
- For every RT-PCR run, one reaction containing CEVd Positive Control (**CEVd *PosC***) and one reaction as no template control (*Nuclease Free-Water*) must be included for proper interpretation of results.
- The recommended minimum number of RNA samples tested per RT-PCR run is 6.
- Using a lower volume from the sample than recommended may affect the sensitivity of CEVd Limit of Detection.

1. Prepare the RT-PCR reaction for sample detection (Set #1, using **CEVd 2X RT-PCR Mastermix**) and the RT-PCR reaction for control detection (Set #2, using **Control 2X RT-PCR Mastermix**) as shown in Table 1 below. The recommended amount of sample RNA to be used is 1 - 2 µL. Ensure that one CEVd detection reaction and one control reaction is prepared for each RNA sample. Adjust the final volume of the RT-PCR reaction to 20 µL using the Nuclease-Free Water provided.

Table 1. RT-PCR Assay Preparation

RT-PCR Components	Volume Per RT-PCR Reaction
CEVd 2X RT-PCR Master Mix Or Control 2X RT-PCR Master Mix	**10 µL**
Sample RNA	**2 µL**
Nuclease-Free Water	**8 µL**
Total Volume	**20 µL**

2. For every RT-PCR run, prepare **one** positive control RT-PCR as shown in Table 2 below:

Table 2. RT-PCR Positive Control Preparation

RT-PCR Components	Volume Per RT- PCR Reaction
CEVd 2X RT-PCR Master Mix Or Control 2X RT-PCR Master Mix	**10 µL**
CEVd *Positive Control (PosC)*	**10 µL**
Total Volume	**20 µL**

3. For every RT- PCR run, prepare **one** no template control RT-PCR as shown in Table 3 below:

Table 3. RT-PCR Negative Control Preparation

RT-PCR Components	Volume Per RT-PCR Reaction
CEVd 2X RT- PCR Master Mix Or Control 2X RT-PCR Master Mix	**10 µL**
Nuclease-Free Water	**10 µL**
Total Volume	**20 µL**

Therefore, at a minimum, each PCR run will contain 6 separate RT-PCR reactions.

C. One Step RT- CEVd PCR Assay Programming

1. Program the thermocylcer according to the program shown in Table 4 below.
2. Run one step RT-PCR.

Table 4. CEVd Assay Program

One Step RT-PCR Cycle	Step	Temperature	Duration
Cycle 1	**Step 1**	**50°C**	**30 min**
Cycle 2	**Step 1**	**95°C**	**3 min**
Cycle 3 (40x)	**Step 1**	**94°C**	**15 sec**
	Step 2	**55°C**	**30 sec**
	Step 3	**72°C**	**45 sec**
Cycle 4	**Step 1**	**72°C**	**5 min**
Cycle 5	**Step 1**	**4°C**	**∞**

D. CEVd One Step RT- PCR Assay Results Interpretation

1. For the analysis of the RT-PCR data, the entire 15-20 µL RT-PCR Reaction should be loaded on a 1X TAE 1.5% Agarose RNA gel along with 10 µL of Norgen's RNA Marker (provided).
2. The RT-PCR products should be resolved on the 1X TAE 1.5% Agarose gel at 150V for 20 minutes (Gel running time will be vary depending on an electrophoresis apparatus).
3. Sample results are provided below:

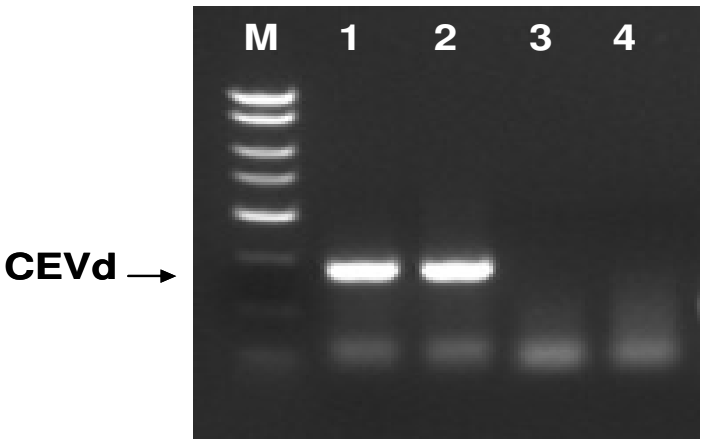

Figure 1: A representative 1X TAE 1.5% agarose gel showing the amplification of CEVd positive (lane 1 and 2) and negative (lane 3 and 4) controls. The size of the CEVd target amplicon corresponds to 270 bp as represented by the provided DNA Marker (M).

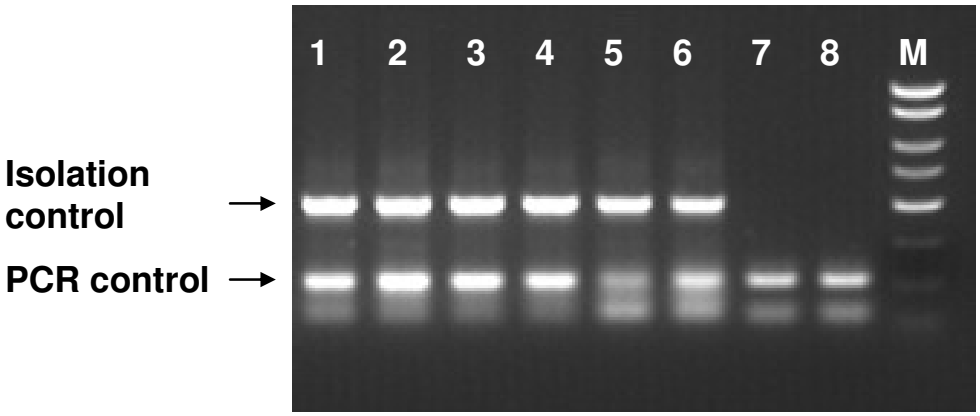

Figure 2: A representative 1X TAE 1.5% agarose gel showing the amplification of **Isolation Control** and **PCR Control** under different conditions using the **Control 2X RT-PCR Mastermix**. The size of the Isolation Control amplicon and PCR Control amplicon correspond to 499 bp and 150 bp, respectively, as represented by the provided DNA Marker (M). Lanes 1 to 6 showed detection of both Isolation Control and PCR Control, suggesting that the RNA isolation as well as the RT-PCR reaction was successful. Lane 7 and 8 showed only the detection of PCR Control suggesting that while the RT-PCR was successful, the isolation failed to recover even the spiked-in Isolation control.

Table 5. Interpretation of One Step RT-PCR Assay Results

Input Type	Target reaction	Control Reaction		Interpretation
	CEVd Target Band (270 bp)	*IsoC* Band (499 bp)	*PCRC* Band (150 bp)	
Positive Control	X	X	X	Valid
Negative Control			X	Valid
Sample	X	X	X	Positive
Sample		X	X	Negative
Sample			X	Re-test
Sample				Re-test
Sample		X		Negative
Sample	X		X	Positive
Sample	X	X		Positive
Sample	X			Re-test

** For results obtained that are not covered in Table 5 above, please refer to the Troubleshooting Section.

E. CEVd RT-PCR Assay Specificity and Sensitivity

- The specificity of Norgen's CEVd RT-PCR Detection Kit is first and foremost ensured by the selection of the CEVd-specific primers, as well as the selection of stringent reaction conditions. The CEVd universal primers were checked for possible homologies to all plant viruses in GenBank published sequences by sequence comparison analysis and published CEVd strains.

F. Linear Range

- The linear range of Norgen's CEVd RT-PCR Detection Kit was determined by analysing a dilution series of a CEVd quantification standards ranging from 100 ag to 1 pg.
- Each dilution has been tested in replicates (n = 4) using Norgen's CEVd RT-PCR Detection Kit on a 1X TAE 1.5% agarose gel.
- The linear range of Norgen's CEVd RT-PCR Detection Kit has been determined to cover concentrations from 100 ag to 1 ng
- Under the conditions of the Norgen's CEVd RNA Isolation procedure, Norgen's CEVd RT-PCR Detection Kit covers a linear range from 100 copies to 1 x 10^6 copies.

Frequently Asked Questions

1. How many samples should be included per RT-PCR run?

- Norgen's CEVd RT-PCR Detection Kit is designed to test 24 samples. For every 6 samples, a non-template control (Nuclease Free Water) and a Positive Control must be included. It is preferable to pool and test 6 samples at a time.

2. How can I interpret my results if neither the CEVd RT-PCR control nor the Isolation Control (*IsoC*) amplifies?

- If neither the CEVd RT-PCR control nor the CEVd Isolation Control (*IsoC*) amplifies, the sample must be re-tested. If the positive control showed amplification, then the problem occurred during the isolation, where as if the Positive control did not amplify, therefore the problem has occurred during the setup of the PCR assay reaction.

3. How should it be interpreted if only the CEVd RT-PCR control showed amplification but neither the CEVd target nor the CEVd Isolation control amplified for a sample?

- This indicates a poor isolation. The isolation procedure must be repeated.

4. How should it be interpreted if only the Isolation Control (*IsoC*) was amplified in a sample?

- The sample tested can be considered as CEVd negative.

5. How should it be interpreted if the CEVd RT-PCR control and the CEVd target showed amplification in a sample?

- The sample tested can be considered positive. It could happen when too much template was added to the reaction.

6. How should it be interpreted if only the CEVd target and the CEVd PCR control were amplified in a sample?

- The sample tested can be considered as CEVd positive.

7. How should it be interpreted if only the CEVd target was amplified in a sample?

- The sample tested should be considered as CEVd positive. At high CEVd input, the CEVd amplicon will be predominant and thus the CEVd PCR control as well as the CEVd Isolation control may not amplify as they compete for PCR resources.

8. How should it be interpreted if only the CEVd RT-PCR control and the Isolation control showed amplification in a sample?

- The sample tested can be considered negative

9. What if I forgot to do a dry spin after my third wash?

- Your first RNA elution will be contaminated with the Wash Solution. This may dilute the RNA yield in your first elution and it may interfere with the PCR detection, as ethanol is known to be a PCR inhibitor.

10. What if I forgot to add the Isolation Control (*IsoC*) during the isolation?

- It is recommended that the isolation is repeated.

Related Products	Product #
Lysis Solution (100 mL)	25806
Plant RNA/DNA Purification Kit	24400
Plant/Fungi RNA Purification Kit	25800
Viroid RNA Purification Kit	32800
Bacterial Genomic RNA Isolation Kit	17900

Technical Assistance

NORGEN's Technical Service Department is staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of NORGEN products. If you have any questions or experience any difficulties regarding Norgen's Citrus Exocortis Viroid (CEVd) RT-PCR Detection Kit or NORGEN products in general, please do not hesitate to contact us.

NORGEN customers are a valuable source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at NORGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please contact our Technical Support Team between the hours of 8:30 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362 or call one of the NORGEN local distributors (www.norgenbiotek.com) or through email at techsupport@norgenbiotek.com.

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